IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Jon Carl Marlowe et al.	Confirmation No.:	9078
Serial No.:	10/734,063	Art Unit:	1631
Filed:	December 10, 2003	Examiner:	Jason M. Sims
For:	AUTOMATED SYSTEM AND METHOD FOR PREPARING AN ASSAY READY BIOLOGICAL SAMPLE	Attorney Docket No.:	9301-232-999
		CAM No.:	301891-999224
		Date: August 7, 2007	

BRIEF ON APPEAL UNDER 35 U.S.C. § 134 AND §§ 41.35 AND 41.37

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This is an appeal under 35 U.S.C. § 134 and §§ 41.35 and 41.37 from a final rejection mailed November 30, 2006 of claims 14-22 of the above-identified application. A Notice of Appeal and Request for Pre-Appeal Brief Conference was filed by Appellants on February 28, 2007. A Notice of Panel Decision from the Pre-Appeal Brief Review ("Notice") was mailed on May 7, 2007, instructing Appellants to proceed to the Board of Appeals and Interferences because there is at least one actual issue for appeal.

The Notice reset the time period for filing this Appeal Brief to one-month from the Notice's mail date of May 7, 2007. Therefore, Appellants request a two-month extension of time from June 7, 2007, to August 7, 2007, to submit this Appeal Brief. Appellants hereby authorize the Commissioner to charge Jones Day Deposit Account No. 50-3013 the $450.00 two-month extension of time fee. Appellants also authorize the Commissioner to charge the Jones Day deposit account for $500.00 for filing a brief in support of the appeal.

I. REAL PARTY IN INTEREST

The real party in interest is Rosetta Inpharmatics LLC as evidenced by an assignment recorded on August 17, 2004, at reel 015693, frame 0334.

II. RELATED APPEALS AND INTERFERENCES

There are no other appeals, interferences, or judicial proceedings known to Appellants, Appellants' legal representative, or assignee, which may be related to, directly affect or be directly affected by, or have a bearing on the Board's decision in the pending appeal.

III. STATUS OF CLAIMS

Claims 1-13 and 23-25 have been canceled.

Claims 14-22 are rejected.

Claims 14-22 are appealed.

IV. STATUS OF AMENDMENTS

All amendments have been entered. None of the pending claims have been amended subsequent to the final rejection dated November 30, 2006.

V. SUMMARY OF CLAIMED SUBJECT MATTER

Claim 14 is the only independent claim pending in this application. The subject matter of independent claim 14 is directed to a computer implemented method for preparing a binding-ready biological sample for a binding assay.

The method includes a step of receiving a binding assay design for a binding assay. This step is described in the original as-filed specification at least at page 5, paragraph [0025] and is shown, for example, at least in FIG. 1, box 100. More specifically, paragraph [0025] states on the 5th and 6th line that "[t]he binding assay design or plan is received, at step 100, by the operator." Also, paragraph [0063] on page 13 of the original as-filed specification states that Figures 4A-4E "are flow charts of a method 400 for preparing binding-ready biological samples" and that initially, "a scientist conceives of and designs a binding assay" and "then sends the binding assay plan or design to the experiment design manager." Similarly, paragraph [0064] on page 13 states that in a preferred embodiment, "the scientist sends the binding assay plan or design to the experiment design manager (Figure 2)."

The method also includes a step of preparing an experiment design for generating a binding-ready biological sample to be used in the binding assay. This step is described in the original as-filed specification at least at page 6, paragraph [0030] and is shown, for example, at least in FIG. 3C, reference numeral 208, Experiment Design Manager. More specifically, paragraph [0030] states on lines 2-5 that "[t]he experimental design manager primarily consists of software that assists a scientist in designing an experiment, stores the experiment design in a database, schedules the execution of experiments, and groups experiments into Robotic Work Units (RWU)." Also, paragraph [0067] on page 14 states that:

> [b]ased on the received binding assay plan or design, the experiment design procedures 349 (Figure 3C) then prepare an experiment design for generating the binding-ready biological sample, at step 403. For example, the experiment design procedures 349 (Figure 3C) determine the processes required to prepare the binding-ready biological sample and the raw materials required to perform these processes.

The method further includes a step of choosing a robot method for generating the binding-ready biological sample. This step is described in the original as-filed specification at least at page 15, paragraph [0072] and is shown at least in FIG. 3C, reference numeral 350, Materials Optimization Procedures. More specifically, paragraph [0072] states on lines 1-5 that "[t]he materials optimization procedures 350 (FIG. 3C) also choose a preferred robot method for generating the binding-ready biological sample, at step 416. A robot method is a file resident on the controller and contains an explicit sequence of robot instructions for preparing a set of binding-ready samples according to a predetermined protocol."

The method also includes a step of generating work instructions for generating the binding-ready biological sample based on the experiment design and the robot method. This step is described in the original as-filed specification at least at pages 16-17, paragraph [0076] and is shown at least in FIG. 3C, reference numeral 350, Materials Optimization Procedures, and FIG. 4B, reference numerals 426 and 427. More specifically, lines 1-4 of paragraph [0076] state that "[i]f there are enough materials (426-Yes), then the materials optimization procedures 350 (FIG. 3C) generate work instructions for producing binding-ready biological samples in accordance with the experiment design, robot method, RWU, and available materials, at step 427."

The method further includes a step of executing the work instructions on robot stations to generate the binding-ready biological sample. This step is described in the original as-filed specification at least at page 8, paragraph [0037] and is shown at least in FIG. 3A, reference numeral 220, Controller. More specifically, lines 1-3 of paragraph [0037]

state that "[t]he controller 220 is coupled between the integration server 218 and a serial splitter 222. The controller 220 is preferably a computer that is used to control and schedule preparation of binding-ready biological samples by the robot stations 204."

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The following grounds of rejection are presented for review in this appeal:

Firstly, whether claims 14-22 are obvious under 35 U.S.C. § 103(a) over U.S. Patent No. 4,780,423 ("Bluestein et al.") in view of U.S. Patent No. 6,996,538 ("Lucas").

VII. ARGUMENT

A. The Applicable Case Law

Section 103 of the Patent Act states that a patent may not be obtained ... "if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains."

The question of obviousness is resolved on the basis of underlying factual determinations including (1) the scope and content of the prior art, (2) any differences between the claimed subject matter and the prior art, (3) the level of skill in the art. *Graham v. John Deere Co.*, 383 U.S. 1, 17-18, 148 USPQ 459, 467 (1966). The Court in *Graham* further noted that evidence of secondary considerations, such as commercial success, long felt but unsolved needs, failure of others, etc., "might be utilized to give light to the circumstances surrounding the origin of the subject matter sought to be patented." 383 U.S. at 18, 148 USPQ at 467.

The Supreme Court ruled in *KSR Int'l Co. v. Teleflex, Inc.* (U.S., 127 S.Ct. 1727, 167 L. Ed. 2d 705 (April 30, 2007)) that the Court of Appeals for the Federal Circuit had applied a too-rigid standard for determining obviousness under Section 103 of the Patent Act and held that the standard for determining obviousness was more expansive and flexible consistent with Supreme Court precedent. According to the Supreme Court, "[w]hen a work is available in one field of endeavor, design incentives and other market forces can prompt variations of it, either in the same field or a different one" and "[i]f a person of ordinary skill can implement a predictable variation, Section 103 likely bars its patentability." Moreover, the Supreme Court stated that "a court must ask whether the improvement is more than the predictable use of prior art elements according to their established functions."

B. The Rejection of Claims 14-22 Should Be Reversed

Initially, Appellants note that Bluestein is directed to heterogeneous fluorescence assays using controlled pore glass particles. More specifically, in Bluestein, a binding-ready biological sample for a binding assay is ferritin. The binding assay is the immunoassay, and the anti-ferritin antibody is a reagent in the binding assay. Appellants respectfully submit that nothing in Bluestein would disclose or suggest to a person of ordinary skill in the art a computer implemented method for **preparing a binding-ready biological sample** (ferritin) for a binding assay (immunoassay). The Bluestein reference only describes an automated method for the immunoassay.

The Office Action mailed November 30, 2006 at page 3, 2nd full paragraph, states that "Bluestein et al. teaches the second step of claim 14 at col. 8, lines 10-31." Appellants respectfully submit that nothing in this section would disclose preparing an experiment design for generating a binding-ready biological sample to be used in said binding assay. Column 8, lines 10-31 of Bluestein describes changes made in the immunoassay protocol, materials, and equipment. For example, in the statement at col. 8, lines 22-24, that "300 ul of solid phase (CPG) anti-ferritin antibody was employed to which was added 150 ul of sample," the sample is already prepared and binding-ready prior to adding the anti-ferritin antibody reagent. These changes pertain to the actual immunoassay, not the sample preparation as recited in claim 14. In addition, the Bluestein reference discloses at column 7, lines 20-25, incubating the solid phase antibody with 200 ul of sample standards. Appellants respectfully submit that this section discloses that the samples are binding-ready and already prepared, i.e., there is no description of preparing the sample as recited in claim 14.

The Office Action at page 3, 3rd full paragraph, states that "Bluestein et al. teaches the third step of claim 14 at col. 8, lines 61-69 and col. 9, lines 1-30. Appellants respectfully submit that nothing in this section would disclose preparing an experiment design for generating a binding-ready biological sample. The Pandex Screen Machine described in these sections automatically perform the immunoassay, i.e., nothing in these sections would disclose preparing a binding-ready biological sample for a binding assay. More specifically, in Bluestein, column 9, lines 17-29, states that "30 ul of the CPG reagent were added to each well of the microtiter plate followed by 30 ul of the standard." The "standard" is the binding-ready biological sample. The standard is already prepared when it is added to the plate. In other words, nothing in Bluestein has been found in these sections or any other sections that would disclose preparing the sample as recited in claim 14.

The Examiner contends that the third step of claim 14 is taught at col. 8, lines 61-69, and col. 9, lines 1-30, of Bluestein, and specifically points to col. 9, lines 8-12 and lines 13-

15. At col. 9, lines 8-12, Bluestein discloses that the Pandex SCREEN MACHINE can be programmed to add reagents and wash solutions for immunoassays (the immunoassays being referred to at col. 9, lines 2-3). Appellants respectfully point out that adding the reagents and wash solutions as recited at col. 9, lines 10-11 is adding, not preparing substances used in the immunoassay. Thus, this recitation does not hint or suggest the generation of binding-ready biological samples (such as might be used in the immunoassay). Moreover, col. 9, lines 13-15, of Bluestein state that the SCREEN MACHINE was used to perform "the 2 site immunometric assay for ferritin described above in Examples 1 and 2." Bluestein further discloses (col. 9, lines 15-16) that the same materials as in Examples 1 and 2 were employed. Examples 1 and 2 describe an embodiment of a well-known immunoassay configuration, i.e., a sandwich immunoassay (see col. 6, lines 39-53). In this sandwich immunoassay, anti-ferritin antibody coupled to glass particles is one end of the "sandwich" (col. 6, lines 61-65), which is then bound to the ferritin (the analyte being measured) in the sample which forms the "center" of the "sandwich" (col. 6, lines 29-30), which in turn is bound to a fluorescein labeled anti-ferritin antibody (col. 6, lines 49-54) (the other end of the "sandwich"). Thus, as would be clear to one of ordinary skill in the art, Bluestein describes a sandwich immunoassay in which anti-ferritin antibody bound to glass particles, and anti-ferritin antibody bound to fluorescein, bind at distinct sites to ferritin in the sample. It is the binding of these three components that is the performance of the immunoassay carried out by the SCREEN MACHINE as taught in col. 9 of Bluestein. Nowhere in Bluestein is there any hint or suggestion of using the SCREEN MACHINE system, or any other method, to generate the binding ready biological samples to be used in the immunoassay. In particular, Bluestein does not hint or suggest using a robot method to prepare the antibody bound to glass particles, the antibody bound to fluorescein, or most significantly, the biological sample containing the analyte ferritin.

Appellants note further that MacCrindle et al. (Clin. Chem. Vol. 31, No. 9:1487-1490 (1985)), reference B1 of record in the Supplemental Information Disclosure Statement filed on August 24, 2006, and attached hereto for the Office's convenience, describes the Pandex machine in greater detail. In particular, the MacCrindle reference at page 1488, 2nd paragraph states: "[t]he samples are diluted with the Digiflex Pipetting Station and pipetted into the Epicon assay plate. Once the reagents and Epicon plates are loaded and the basic instrument parameters are selected, all further operations are completely automatic...." The samples are clearly binding-ready and prepared prior to adding to the Epicon plates and loading into the Pandex machines.

Moreover, the MacCrindle reference at page 1488, 3rd paragraph states: "[u]sing the Digiflex pipettor, we make two serial dilutions of the sample...Fifty microliters of this dilution of sample is pipetted into a well of the Epicon assay plate. Then, to each well the Screen Machine adds 20 μL of the 2.5 mg/mL suspension of antibody-coated particles...." Again, this section clearly describes that the samples are prepared and binding ready prior to processing by the Screen Machine. The Screen Machine only performs the immunoassay – i.e. adding the reagents, incubations, washes (see 2nd paragraph p. 1488 of MacCrindle).

Moreover, the Office Action at page 6, first paragraph states that "[e]xamples 1, 2, and 3 all have automated preparations and these examples describe the automated assay experiments being performed and specifically disclose the preparation of adding the controlled pore glass (CPG) antibody and adding the fluorescein labeled anti-ferritin antibody to each tube, which represents 'a robot method for generating said binding-ready biological sample' because of the automated processes." Appellants submit that examples 1, 2, 3 do not have automated preparations of binding-ready biological samples. The examples are silent on how the samples are prepared prior to being added to the immunoassay as binding-ready samples. The adding of controlled pore glass (CPG) antibody and fluorescein labeled anti-ferritin antibody are steps in an immunoassay, i.e. adding the reagents to the binding-ready biological sample (ferritin), and does not represent "a robot method for generating said binding-ready biological sample."

The Office Action at page 6, last paragraph also states that "[t]he automated deposit of the CPG antibody to each tube followed by the deposit of the fluorescein labeled anti-ferritin antibody to each tube demonstrates the preparation of a binding-ready biological sample." Appellants respectfully submit that the CPG antibody and fluorescein labeled anti-ferritin antibody additions are components in a binding assay, i.e., these steps deal with delivering the components so as to carry out the binding assay, not with the preparation of the components. Thus, they do not deal with preparing a binding-ready biological sample as required by claim 14. These antibodies are added to a binding-ready biological sample. Both the CPG anti-ferritin antibody and the fluorescein labeled anti-ferritin antibody bind to the binding-ready biological sample (ferritin). Addition of binding reagents to a binding-ready biological sample is not the preparation of a binding-ready biological sample.

Appellants note for the record that the Office Action rejected claims 14-22 under 35 U.S.C. § 103(a) as obvious over Bluestein et al. in view of Lucas. Appellants submit that Lucas discloses an inventory control system and methods which allow third parties to monitor company inventory via the Internet and World Wide Web, and automatically order needed items. Appellants submit that Lucas would not remedy any of the deficiencies

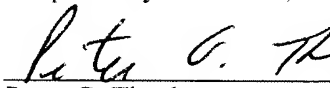
discussed above in regard to Bluestein since it is directed to an inventory control system and would not teach a person of ordinary skill in the art a computer implemented method for preparing a binding-ready biological sample for a binding assay that includes the steps recited in claim 14. In fact, the Examiner did not use Lucas in the Office Action to reject any features recited in claim 14, i.e., the Examiner first refers to Lucas in the Office Action to reject the feature recited in claim 16.

In regard to the Supreme Court's KSR decision in relation to the pending claims, the Supreme Court, as mentioned above, stated that "[i]f a person of ordinary skill can implement a *predictable* variation [over the prior art], Section 103 likely bars its patentability." (emphasis added.) Appellants submit that Bluestein and Lucas do not teach that a computer implemented method for preparing a binding-ready biological sample for a binding assay is predictable, when these references are considered in combination or separately. Therefore, even in light of the Supreme Court's KSR decision, Appellants submit that claim 14 is patentable over the cited prior art.

For all the reasons set forth above, Appellants respectfully request that the Section 103(a) rejection of claims 14-22 based on Bluestein in view of Lucas be reversed.

Respectfully submitted,

Date: August 7, 2007



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VIII. CLAIMS APPENDIX

CLAIMS UNDER APPEAL
U.S. APPLICATION NO. 10/734,063
ATTORNEY DOCKET NO. 9301-232

14. A computer implemented method for preparing a binding-ready biological sample for a binding assay, comprising:

receiving a binding assay design for a binding assay;

preparing an experiment design for generating a binding-ready biological sample to be used in said binding assay;

choosing a robot method for generating said binding-ready biological sample;

generating work instructions for generating said binding-ready biological sample based on said experiment design and said robot method; and

executing said work instructions on robot stations to generate the binding-ready biological sample.

15. The method of claim 14, further comprising, before said generating, optimizing materials usage and plate layout for generating said binding-ready biological sample.

16. The method of claim 14, further comprising, before said generating, checking inventory for materials required for said experiment design.

17. The method of claim 16, wherein said checking comprises:

sending a inventory request to an inventory system;

receiving a list of all materials available in inventory;

ascertaining whether there are enough materials in inventory for said preparation.

18. The method of claim 16, wherein said checking comprises:

sending a inventory request to an inventory system, where said inventory request contains a list of materials required for said preparation;

receiving inventory data indicating whether said materials are available in inventory; and

ascertaining from said inventory data whether said materials are available in inventory.

19. The method of claim 18, wherein said ascertaining comprises:

concluding that there are not enough materials in inventory for said preparation;

notifying an operator that there are insufficient materials in inventory; and

repeating said ascertaining until there are enough materials in inventory for said preparation.

20. The method of claim 14, wherein said receiving further comprises acquiring a tissue sample.

21. The method of claim 20, further comprising, after said acquiring:

extracting a constituent sample from said tissue sample; and

updating inventory to include said constituent sample.

22. The method of claim 14, wherein said binding-ready biological sample is a hybridization-ready biological sample, and said binding assay is a hybridization assay.

IX. EVIDENCE APPENDIX

None

X. RELATED PROCEEDINGS INDEX

None

CLIN. CHEM. 31/9, 1487–1490 (1985)

Particle Concentration Fluorescence Immunoassay: A New Immunoassay Technique for Quantification of Human Immunoglobulins in Serum

Chris MacCrindle, Kathryn Schwenzer, and Michael E. Jolley

A new fluorescence immunoassay technique, particle concentration fluorescence immunoassay (PCFIA), has been developed for quantifying the human immunoglobulins (IgA, IgM, and IgG). In these "two-site sandwich assays," the capture antibody is immobilized on small polystyrene spheres and the tracer is fluorescein-labeled antibody. Polystyrene particles less than 1 μm in diameter make up the solid phase, to which goat anti-human antibody for each respective assay is attached. Serum specimens are diluted (5000-fold for IgA or IgM, 20 000-fold for IgG) placed on the 96-well Pandex assay plate; and mixed with the solid phase and tracer (fluorescein-labeled goat anti-human IgA, IgM, or IgG), which are added automatically by the Pandex Screen Machine™. This instrument incubates the reaction mixture for 17 min at ambient temperature, separates the bound and free label by filtration, washes the solid phase, and determines the total particle-bound fluorescence by front-surface fluorimetry or epifluorescence, calculates results, and generates detailed reports. Ninety-six specimens may be analyzed in 29 min or 960 specimens in 136 min. Results by PCFIA for IgA, IgM, and IgG in serum correlated well with those by rate nephelometry.

Additional Keyphrases: *IgA · IgM · IgG · proteins · rate nephelometry compared · dual-wavelength fluorometry*

Current methods of choice for diagnosing immunoglobulin (Ig) abnormalities are immunoelectrophoresis or serum protein electrophoresis, combined with quantification by nephelometry, radial immunodiffusion, or enzyme-linked immunosorbent assay. These techniques provide adequate results, but less-expensive methods are needed to rapidly quantify and characterize protein abnormalities, in particular the Ig disorders (*1*). A new fluorescence immunoassay technique, particle concentration fluorescence immunoassay (PCFIA) (*2*), offers the specificity of immunoassay and rapid batch quantification of human IgA, IgM, and IgG.[1] In PCFIA, immunoreactive species are bound to small particles, typically 0.8-μm-diameter polystyrene; the resulting solid phase is dispersed in the sample, and labeled reagent is added. After analyte and labeled reagent bind on the surface of the particles, the reaction mixture is filtered, washed, and the total particle-bound fluorescence determined. By using a reaction well with a 2-mm-diameter filtration area (Figure 1), the fluorescence from all of the solid phase may be detected. Background fluorescence, primarily from stray and scattered light and from the endogenous fluorescence of the filter membrane and solid phase, can be minimized by good optical design (use of filters with narrow band widths) and careful selection of materials to be used in, e.g., the filter membrane, the assay plate, and the solid phase.

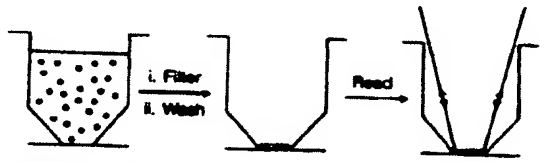

Fig. 1. Principle of PCFIA

Antibody-coated polystyrene particles and fluorescent-labeled antibody reagent are added to an assay well containing the sample. When the reaction is complete, the mixture is filtered through a membrane at the bottom of the well. Particles with bound fluorescent label are concentrated on top of the membrane, unbound substances are filtered away. Particles are concentrated on a 2-mm-diameter filter, where 100% of the available fluorescence emission is detected

Materials and Methods

Materials

Polystyrene latex particles, approximately 0.6–0.8 μm in diameter, are commercially available from Pandex Laboratories, Mundelein, IL 60060. Polyclonal antibody (goat antibody to human IgA, IgM, or IgG) was obtained from Southern Biotechnology Associates, Inc., Birmingham, AL 35226, as were tracers, fluorescein isothiocyanate (λ_{ex} 485 nm/λ_{em} 535 nm)-labeled goat anti-human IgA, IgM, or IgG.

Polyclonal antibody was passively adsorbed to the polystyrene latex particles at an antibody concentration of 100 μg/mL and a particle concentration of 5 mg/mL in 20 mL of 0.1 mol/L sodium acetate buffer, pH 4.0. After letting the reaction mixture stand for 2 h, with periodic vortex-mixing, we centrifuged the particles at approximately 3000 × *g* for 15 min, then decanted the supernate. We resuspended the particles in 40 mL of isotonic buffered saline (IBS) (American Scientific Products, McGaw Park, IL 60085), re-centrifuged, and again decanted the supernate. The final suspension of particles was at 2.5 mg/mL in 40 mL of IBS. Purple reference particles (λ_{ex} 590 nm/λ_{em} 620 nm; Pandex cat. no. 30-030-1), which serve as an internal fluorescent reference standard, were added to the antibody-coated particles at a final concentration of 50 μg/mL.

Standards were prepared by diluting the Beckman Immunochemistry System (ICS) Calibrator I (lot C405022); Beckman Instruments, Inc., Brea, CA 92821) in "1% diluent": per liter, 10 mL each of fetal calf serum (Biolabs, Chicago, IL 60045) and normal goat serum (Miles Scientific, Naperville, IL 60566) in IBS. Other standards and controls were obtained from the following sources: the Centers for Disease Control, Atlanta, GA 30333; College of American Pathologists, Skokie, IL 60077-3388; Syva Co., Palo Alto, CA 94303; Beckman Instruments Inc.; Abbott Laboratories, North Chicago, IL 60046; Kallestad, Austin, TX 78701; Hyland, Division of CooperBiomedical, Malvern, PA 19355; Kent,

Pandex Laboratories, 909 Orchard St., Mundelein, IL 60060.

[1] Nonstandard abbreviations: PCFIA, particle concentration fluorescence immunoassay; FITC, fluorescein isothiocyanate; IBS, isotonic buffered saline.

Received April 11, 1985; accepted June 14, 1985.

Redmond, WA 98053; and Tago, Burlingame, CA 94010. Compounds used to test cross-reactivity were obtained commercially (purified IgA and IgM from Pel Freeze Biologicals, Rogers, AR 72756, and purified IgG from Cappel, Division of CooperBiomedical).

Special Apparatus

A special microprocessor-controlled fluorimeter designed to measure epifluorescence was constructed in our laboratories (Screen Machine™, Pandex Laboratories). [In epifluorescence the emitted light passes through the same optical path as the excited light.] The samples are diluted with the Digiflex™ Pipetting Station (Micromedic Systems, Inc., Horsham, PA 19044) and pipetted into the Epicon™ assay plate (Pandex Laboratories). Once the reagents and Eipcon plates are loaded and the basic instrument parameters selected, all further operations are completely automatic: e.g., positioning the assay plate, pipetting reagents and wash solutions, and concentrating the solid phase. All unbound label and extraneous liquid is drawn by negative pressure through a special, plate-mounted filter membrane within each Epicon plate. The plate is then automatically positioned below the optics and the epifluorescence signal is detected by a photomultiplier tube (Figure 1). To ensure consistency of test results, dye-impregnated reference particles are included in the solid-phase suspension. The instrument's dual-wavelength mode automatically ratios the two emission wavelengths of the assay label and the reference dye (in this case 535 nm/620 nm) to minimize such sources of variation as differences between plates and between well positions and variations in lamp intensity. The filter packs are equipped with user-specified excitation and emission interference filters, each optimized for the two fluorescent labels. The ratio of the intensity at two wavelengths is plotted as a function of concentration of the standards. The concentration of a clinical specimen is determined by interpolating between standards.

Procedures

The IgA and IgM assays are performed as follows: Using the Digiflex pipettor, we make two serial dilutions of sample. First, 7 μL of sample is aspirated and dispensed into a dilution vial followed by 493 μL of 1% diluent. For the second dilution, 7 μL of the first dilution is aspirated and similarly diluted with 493 μL of 1% diluent. Fifty microliters of this dilution of sample is pipetted into a well of the Epicon assay plate. Then, to each well the Screen Machine adds 20 μL of the 2.5 mg/mL suspension of antibody-coated particles (solid phase) and 20 μL of diluted tracer (antibody labeled with fluoresecein: 5 μg/mL for IgA, 10 μg/mL for IgM, and 4 μg/mL for IgG). The plate is incubated at ambient temperature for 17 min, after which the liquid in the wells is vacuumed for 1.0 min, the plate is washed (50 μL of IBS per well), and the wash vacuumed for 1.0 min. The instrument then records the fluorescence passing through the fluoresecein filter, with reference to the Texas Red filter, to calculate the ratio of the intensities of each filter.

The IgG assay is performed identically to the IgA and IgM assays, except that the two sample dilutions are greater: first, 7 μL of sample is diluted with 993 μL of 1% diluent; second, 7 μL of the first dilution is diluted with 993 μL of 1% diluent. Thirty microliters per well of this second dilution of sample is pipetted into assay plate.

For comparison, we assayed samples by rate nephelometric immunoassay, using a Beckman ICS with ICS reagents according to the manufacturer's instructions. Correlations between methods were determined by linear regression analysis.

Results

Standard Curves

Figure 2 illustrates typical standard curves for IgA, IgM, and IgG by PCFIA. The IgA standard curve exhibits a "hook" or prozone effect (the ratio of the intensity decreases) at a concentration greater than 1200 mg/dL. The IgM standard curve exhibits a hook effect at concentrations greater than 1400 mg/dL, the IgG standard curve at concentration greater than 4500 mg/dL.

The antibody-coated particles are stable for one week at both 37 °C and 45 °C, and are also stable to a freeze–thaw cycle. Given our previous experience with reagent stability at warmer temperatures, the solid phase should be stable for at least two years at 2–8 °C. The current FITC tracers, however, will not be similarly stable 2–8 °C in the diluted format, and we suggest that they be freshly diluted before use.

Incubations times may be minimized by increasing the concentrations of tracer and analyte in the reaction mixture. In the IgA assay, changing the sample dilution from 5000- to 2000-fold and increasing the tracer concentration from 5 to 30 μg/mL yielded identical standard curves for incubation periods of 7, 10, or 30 min.

Analytical Variables

Specificity. The affinity-purified antibodies were monospecific as determined by various methods. All of the antibodies were tested with purified immunoglobulins by PCFIA. Antibody to IgA, IgM, and IgG used to coat the particles and the FITC-labeled antibodies to IgA, IgM, and IgG were monospecific as determined by double diffusion. All of the antibodies except FITC-labeled anti-IgG were also tested by immunoelectrophoresis. The antibodies to IgA, IgM, and FITC-labeled antibody to IgA were tested by solid-phase RIA with a normal immunoglobulin panel and a myeloma panel.

Analytical recovery. We determined analytical recovery by assaying commercially available standards and controls from several sources, measuring all three immunoglobulins (IgA, IgM, and IgG) by PCFIA. The correlations and slopes (Figure 3) approximately equal 1.0, demonstrating very good recovery in the immunoglobulin assays, especially within the normal range. We also supplemented 10 IgA-containing clinical specimens with 1050 mg of IgA per liter, and measured an average recovery of 93.71% (984, SD 111, mg/L). The average recovery was 101.60% (569, SD 59, mg/L) for 10 IgM-containing specimens supplemented with 560 mg of IgM per liter, and 101.29% (5310, SD 628, mg/L) for 10 IgG-containing specimens with 5240 mg of added IgG per liter.

Sensitivity. Sensitivity, the lowest concentration that can be detected with 95% confidence, was determined by assaying five replicates of the zero standard. We calculated the sensitivity as that concentration detectable at 2 SD from the mean for the zero standard. Our results for this analytical system were as follows: 14 mg/dL for IgA, 10 mg/dL for IgM, and 11 mg/dL for IgG.

Precision. We determined within-assay-plate reproducibility by running eight replicates of low, medium, and high controls for each immunoglobulin (Table 1). The within-plate CVs were <10% for all the assays. We currently (April 1985) recommend including a standard curve on every assay plate with the unknown samples.

Also, the PCFIA system includes a fluorescent internal standard in the antibody-coated particle solution, which compensates for errors in pipetting particles, differences in assay plates, differences between well positions, and varia-

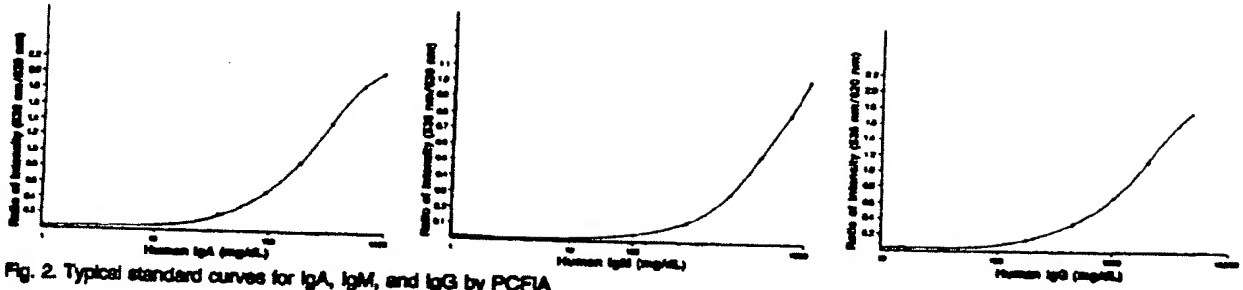

Fig. 2. Typical standard curves for IgA, IgM, and IgG by PCFIA

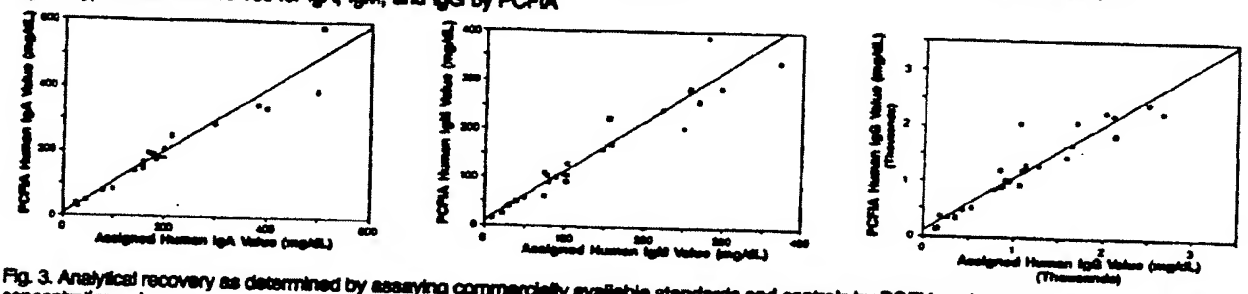

Fig. 3. Analytical recovery as determined by assaying commercially available standards and controls by PCFIA and comparing results with assigned concentration values

The regression equation for IgA is PCFIA = 0.983 assigned value +8.65 (n = 22, r = 0.947, PCFIA average = 212 mg/dL, assigned average = 211 mg/dL). For IgM, PCFIA = 0.99 assigned value +13.5 (n = 25, r = 0.980, PCFIA average = 148 mg/dL, assigned average = 136 mg/dL). For IgG PCFIA = 0.947 assigned value +135.09 (n = 28, r = 0.948, PCFIA average = 1241 mg/dL, assigned average = 1167 mg/dL)

Table 1. Precision

	Concn, g/L		
	Mean	SD	CV, %
IgA	5.60	0.12	2.15
	3.64	0.13	3.52
IgM	1.27	0.02	1.68
	3.23	0.08	2.62
	1.83	0.02	0.93
IgG	0.60	0.01	1.24
	25.14	2.46	9.85
	15.50	1.03	6.66
	5.23	0.31	5.93

tions in lamp intensity. To demonstrate the improved precision obtained by referencing the fluorescein signal to the signal from the reference particles, we calculated the CVs for the fluorescein signal for the IgA assay by using the same standard curve and controls as the CVs calculated from the signal ratio. The fluorescein signal CV for the 560.25 mg/dL control was 8.93%, for the 364.37 mg/dL control 11.74%, and for the 126.62 mg/dL control 5.14%. By comparison with the CVs in Table 1, the addition of the internal standard clearly improves the precision of the PCFIA method.

Correlation with commercially available assays. Figure 4 summarizes the correlation between PCFIA results for IgA, IgM, and IgG and those measured by the Beckman ICS. The correlations, slopes, intercepts, and averages between the PCFIAs and the Beckman ICS assays indicate that PCFIA is a clinically useful tool for determining immunoglobulin concentrations in clinical specimens. With the PCFIA 96 tests may be run in 29 min and 10 plates (960 tests) may be run in 136 min.

Discussion

Determining IgA in 40 specimens with the Beckman ICS requires approximately 30 min of attended time; the PCFIA analysis requires a similar amount of attended time, because of the need for specimen dilution. Total assay time for 40 specimens is approximately 2 h with the Beckman ICS, whereas the PCFIA requires 29 min. As the number of specimens increases, the time saved by PCFIA analysis increases (i.e., 960 tests may be completed in 136 min). If it is desirable to increase the speed of reaction, incubation times for PCFIA analysis of the immunoglobulins can be decreased from 17 to 7 min by increasing the concentration of the tracer and decreasing the dilution of the specimen, so that 40 specimens can be assayed in 19 min.

The PCFIA immunoassay technique is rapid for large batch analysis, and is sensitive and accurate. The polysty-

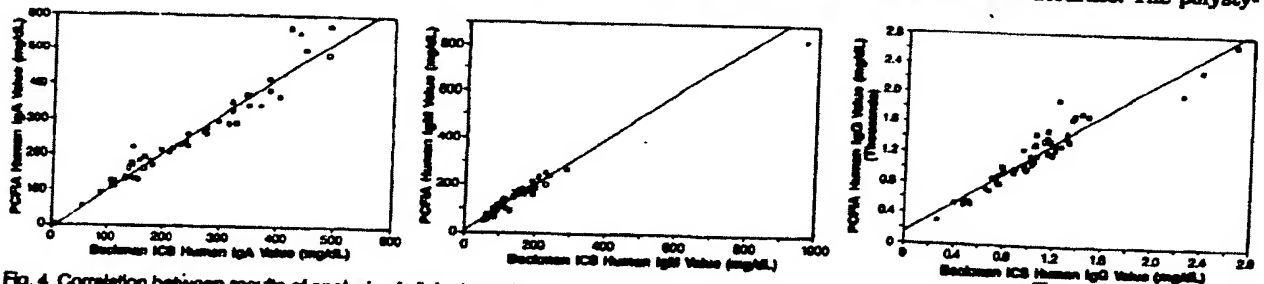

Fig. 4. Correlation between results of analysis of clinical specimens containing immunoglobulin (IgA, IgM, and IgG) by PCFIA and the Beckman ICS

The regression equation for IgA is PCFIA = 1.08 ICS −8.21 (n = 50, r = 0.969, PCFIA average = 249.70 mg/dL, ICS average = 236.08 mg/dL). For IgM, PCFIA = 0.979 ICS + 8.27 (n = 54, r = 0.975, PCFIA average = 166.70 mg/dL, ICS average = 161.77 mg/dL). For IgG, PCFIA = 0.982 ICS +152.98 (n = 55, r = 0.961, PCFIA average = 1192.90 mg/dL, ICS average = 1058.47 mg/dL)

rene particles allow for faster reaction rates than do other technologies: their high surface-area-to-volume ratio and their natural brownian motion keep the reagents and analytes continuously mixing and suspended in solution for an extended period.

The large dilutions of patient's serum (5000- and 20 000-fold) required for these assays demonstrate the high sensitivity of the system, especially for high-molecular-mass analytes, which, until now, have been the domain of enzyme immunoassay or solid-phase RIA. We believe that any possible interferences in the immunoglobulin assays will be negligible at the large dilutions used. In another assay system under development in our laboratories (unpublished data), in which 20 μL of a 50-fold dilution of specimen is used, we saw no problems with icteric, hemolytic, or lipemic specimens. The good precision, recovery, and correlation with an existing method for quantifying immunoglobulins demonstrate the accuracy and clinical utility of PCFIA.

References

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2. Jolley ME, Wang CJ, Ekenberg SJ, et al. Particle concentration fluorescence immunoassay (PCFIA): A new, rapid immunoassay technique with high sensitivity. *J Immunol Methods* 67, 21–35 (1984).